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Chitin synthase III: Synthetic lethal mutants and "stress related" chitin synthesis that bypasses the *CSD3/CHS6* localization pathway

(*Saccharomyces*/cell wall/glucan synthase)

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ABSTRACT **We screened *Saccharomyces* strains for mutants that are synthetically lethal with deletion of the major chitin synthase gene *CHS3*. In addition to finding, not surprisingly, that mutations in major cell wall-related genes such as *FKS1* (glucan synthase) and mutations in any of the Golgi glycosylation complex genes (*MNN9* family) are lethal in combination with *chs3*Δ, we found that a mutation in Srv2p, a bifunctional regulatory gene, is notably lethal in the *chs3* deletion. In extending studies of *fks1*-chitin synthase 3 interactions, we made the surprising discovery that deletion of *CSD3/CHS6*, a gene normally required for Chs3p delivery and activity *in vivo*, was not lethal with *fks1* and, in fact, that lack of Csd3p/Chs6p did not decrease the high level of stress-related chitin made in the *fks1* mutant. This finding suggests that "stress response" chitin synthesis proceeds through an alternate Chs3p targeting pathway.**

The cell wall of the budding yeast, *Saccharomyces cerevisiae*, is composed primarily of mannoproteins, β-1,3 glucan, β-1,6 glucan, and chitin. In vegetative cells chitin, a fibrous polymer of *N*-acetylglucosamine, is found in a ring at the base of the emerging bud, at the septum, and in the lateral wall. Although chitin makes up only 1–2% of the dry weight of the cell wall it is important for cell wall integrity (1). Mutations that affect chitin synthesis cause osmotic sensitivity (2, 3), abnormal morphology, aggregation, and growth arrest with elongated buds (4–6).

There are three chitin synthase genes in *S. cerevisiae*: *CHS1*, (7), *CHS2* (8), and *CHS3* (4). Chs1p is thought to be a repair enzyme, synthesizing chitin in response to an acid-induced increase in chitinase activity after separation of mother and daughter cells (9). Chs2p is localized to the mother-bud junction and functions to synthesize chitin in the primary septum (5, 6, 8). The *CHS3* gene encodes the catalytic subunit of the major chitin synthase CSIII, which is responsible for the synthesis of more than 90% of the chitin in the cell wall (2, 5, 6).

Additional chitin synthesis-related genes have been identified that are required for chitin synthase III activity. The product of the *CHS4* (*CSD4*, *CAL2, SKT5*) gene is required for chitin synthase III activity both *in vivo* and *in vitro* (2, 10). The Chs4p may have a dual role, as a limiting subunit of a Chs3p-containing complex and as an activator of Chs3p (11). In addition, Chs4p links Chs3p to the septins, specifically those encoded by *CDC10*, through Bni4p (12). Chs5p (Cal3p) also is required for chitin synthase III activity both *in vivo* and *in vitro* (13, 14). Chs5p may play a similar role to Chs4p or may function in the localization of Chs3p. Santos *et al.* (13, 14) found that in the absence of Chs5p, Chs3p fails to localize to the neck or the site of the incipient bud. Csd3/Chs6p is required for chitin synthesis *in vivo*, but not for chitin synthase III activity *in vitro* (2, 11) and may be involved in transport of newly synthesized Chs3p from chitosomes to the plasma membrane (15, 16). The recently discovered Chs7p (17) is responsible for export of Chs3p from the endoplasmic reticulum.

The cell wall is a structure that is constantly undergoing change throughout the cell cycle and in response to external stimuli. Chitin synthase III is responsible for the synthesis of chitin during bud emergence and growth, mating, and spore formation (2). Chitin is deposited in a ring, at the site of bud emergence, late in the G_1 phase of the cell cycle. It has been shown by labeling with wheat-germ agglutinin conjugated to FITC as well as staining with Calcofluor that the lateral walls of mother cells contain much more chitin than the lateral walls of buds (5, 6, 18). The chitin in the lateral walls also is made by Chs3p, is cell cycle regulated, and occurs, for the most part, in the later stages of the cell cycle. In addition, Pammer *et al.* (19) found that transcript levels of *CHS*3 do not change significantly during the cell cycle, indicating that Chs3p is regulated posttranscriptionally. These findings indicate that the synthesis of chitin is regulated both spatially with respect to morphogenesis and temporally with respect to the cell cycle.

A synthetic lethal mutant screen is useful in identifying functionally related proteins, interacting proteins, regulatory proteins, and proteins that belong to the same complex (20, 21). Bender and Pringle (22) used a synthetic lethal screen to identify two new genes involved in bud emergence, *BEM1* and *BEM2*, by screening for mutants that required plasmid-borne *MSB1* for viability. Costigan *et al.* (23) used a similar screen to identify *SLK1*, a gene that when mutated requires *SPA2* for vegetative growth. Both *SPA2* and *SKL1* play a role in polarized cell growth. Because deletions of *CHS*3 are viable, we screened for genes that when mutated are lethal only in combination with a *chs*3 deletion.

MATERIALS AND METHODS

Yeast Strains, Media, and Plasmids. Yeast were grown in either rich medium (yeast extract/peptone/dextrose, YPD) or in synthetic minimal (SD) that have been described (24). Other media were SD^+ (SD plus adenine, histidine, uracil, leucine, lysine, and tryptophan), SD^+ −ura (as SD^+ but lacking uracil), SD^+ −trp (as SD^+ but lacking tryptophan), and SD^+ −leu −trp (as SD but lacking both leucine and tryptophan). Calcofluor white-containing medium was made as described (2).

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Abbreviations: YPD, yeast extract/peptone/dextrose; SD, synthetic minimal medium.

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SD^+ medium was used for all drug tests with the exception of Nikkomycin Z. For Nikkomycin Z drug tests, SDA^+ medium, which contains allantoin (1 mg/ml final concentration) as the nitrogen source, was used (25). Solid media contained 2% agar (Difco), unless otherwise noted. All strains were grown at 26°C.

Strain Construction. Standard procedures of yeast genetics were used (24). Yeast transformations were done by using the lithium acetate method (26). Standard methods were used for the construction of plasmids. *Escherichia coli* strain DH5α was used for transformation and plasmid construction. *E. coli* were transformed by using the procedure of Inoue *et al.* (27).

Plasmids. *pBK101.* The 3.7-kb *Bam*HI/*Nhe*I fragment of pDK255 (28) containing *ADE3* was cloned into the *Bam*HI/*Spe*I sites in the multiple cloning site of the *TRP1*-marked *CEN*6 vector pRS314 (29) to make pBK101.

pBK102. pCSD2–15, a 5.5-kb *Cla*I-*Bam*HI fragment of pCSD2–3 cloned into pSK (Stratagene), was cut with *Bam*HI and *Sal*I. The 5.5-kb band containing *CHS3* (*CSD2*) was gel purified and ligated to *Bam*HI/*Sal*I-digested pBK101 to make pBK102.

pCSD2–3. Bulawa (2) subcloned the 5.4-kb *Cla*I–*Bam*HI fragment of pCSD2 into the same sites of the *CEN*6/*ARSH*4 vector pRS316 (29).

pRS316. URA3-marked *CEN6/ARSH4* vector was constructed as described (29).

p12a-1. This plasmid (derived from a *CEN4 URA3* genomic library) complements *SRV2* mutants.

p13d-3. This plasmid (derived from a *CEN4 URA3* genomic library) complements *FKS1* mutants.

p13a-1. This plasmid (derived from a *CEN4 URA3* genomic library) complements *ANP1* mutants.

Yeast Genomic Library. The *CEN*4 *URA*3-marked yeast genomic library was a generous gift from the Young Laboratory (Whitehead Institute, Cambridge, MA) and is described elsewhere (30).

Synthetic Lethal Screen. Strain PRY487 was grown on SD^+-leu$-$trp. Individual colonies were suspended in 2 ml of SD^+-leu$-$trp and grown for 90 min at 26°C. A 1-ml aliquot of each suspension was sonicated briefly to disperse clumps. Cell counts were done by using a hemocytometer. Suspensions were diluted in H_2O and plated at 2×10^3 cells/plate on YPD. These cells (total of 1×10^5 cells) were mutagenized with UV irradiation to a viability of 6% (60-sec exposure, 40 cm from source, lamp output 10 erg/mm^2), and lids were replaced and incubated at 26°C in the dark.

Characterization of Putative Synthetic Lethal Mutants. Standard genetic procedures were used to determine the recessiveness or dominance of the mutants. Nonsectoring $Sect^-$ mutants were mated to PRY 398, and the resulting diploids were tested for their ability to sector by streaking on YPD. Tetrad analysis was performed on diploids whose sectoring phenotype was recessive to confirm that both $Sect^-$ and $Sect^+$ were recoverable. This analysis also served to backcross the mutagenized strains.

Putative synthetic lethals were transformed with pRS316 and the *CEN* plasmid pCSD2–3, which carries a wild-type copy of *CHS3* to verify the requirement for the plasmid-borne *CHS3* for viability.

Agar Diffusion Assay for Drug Sensitivity. Agar diffusion assays were used to test synthetic lethal mutants for sensitivity to the following drugs: Hygromycin B, Amphotericin B, Nikkomycin Z, tunicamycin, the Echinocandin L-733,560, sodium orthovanadate, and FK-506. The procedure used was a slightly modified version of that described by Island *et al.* (25).

Cloning of the Gene Complementing the $Sect^-$ Phenotype. We used the drug sensitivity of the synthetic lethal strains to facilitate the cloning of the genes, which, when mutated, are synthetically lethal with a *CHS3* deletion. Synthetic lethal strains hypersensitive to Nikkomycin Z were transformed with the *CEN4 URA3* library and plated on SD^+-ura$-$trp at a density of 1×10^3 cells/plate. After 3–4 days, transformants were replica plated to SDA^+ $-$ura$-$trp, plus 15 mg/ml Nikkomycin Z. After 1–2 days incubation at 26°C, any transformants that had grown on the Nikkomycin Z plate were streaked for singles on YPD and YPD plus 500 mg/ml Calcofluor white. Single colonies were retested for the presence of the plasmid(s) and the ability to grow on plates with 15 mg/ml Nikkomycin Z. Transformants that were able to sector on YPD and resistant to Calcofluor and Nikkomycin Z underwent plasmid rescue (31).

Synthetic lethal strains that were sensitive to Calcofluor white were transformed with the *CEN4 URA3* library and plated on SD^+ $-$ura plates as described above. The SD^+-ura plates contain tryptophan so there is no auxotrophic requirement for maintaining the *TRP1*-marked plasmid carrying the *CHS3* gene. After 3–5 days, colonies were replica-plated onto SD^+ $-$ura plus 700 mg/ml Calcofluor white. Calcofluor-resistant colonies from library-transformed strains were restreaked on YPD, YPD plus 700 mg/ml Calcofluor, SD^+ $-$ura, SD^+ $-$trp, and SD^+ plus 1 mg/ml 5-fluoroorotic acid (32). Complementing library plasmid DNA was prepared from the yeast strain by the method of Hoffman and Winston (31).

Cell Wall Assays. Chitin was measured by the method described in Bulawa (2). Other cell wall assays were done by using a slightly modified version of the procedure described by Castro *et al.* (33).

β-Glucan Synthase Assays. Enzyme preparation and assays were done as described with minor modifications (33). Briefly, early logarithmic cells were resuspended in 1 mM EDTA (pH 8) and lysed in a BeadBeater. The crude lysate was centrifuged at low speed to remove unbroken cells and cell wall debris. After a high-speed spin, washed pellets were resuspended in buffer plus glycerol and stored at -20°C. β-1,3 Glucan synthase reactions were done as described (33). The amount of [^{14}C]-glucose incorporated into acid-insoluble glucan was determined by using a Millipore filter method. The amount of [^{14}C]-glucose incorporated into insoluble glucan trapped on the filter was determined by liquid scintillation counting.

RESULTS

Characterization of Putative Synthetic Lethal Strains. To show that the requirement for the plasmid is caused by the presence of *CHS3* and not by other sequences on the plasmid, putative synthetic lethal mutant strains were transformed with pRS316, a *URA3*-marked plasmid having a backbone identical to that of pBK102. Transformation with pRS316 should not effect the $Sect^-$ phenotype of true synthetic lethals because it does not carry *CHS3*.

Putative synthetic lethal strains also were transformed with the *CEN* plasmid pCSD2–3, which carries a wild-type copy of *CHS3* but not *ADE3.* True synthetic lethals should sector when they acquire pCSD2–3 because either plasmid can satisfy the requirement for *CHS3.* Of the 23 putative synthetic lethal mutant strains tested, 10 behaved as defined for true synthetic lethals when transformed with the tester plasmids. Of these 10, four mutants grew very slowly (1b-1, 1d-1, 5c-2, 7e-1) and one mutant, (9c-5) failed to grow in top agar. For the present study, the remaining five mutant strains were further characterized.

Agar Diffusion Assay for Drug Sensitivity. The five synthetic lethal mutant strains that were recessive and sectored only when transformed with a plasmid carrying *CHS3* were tested for growth in the presence of cell wall-specific drugs. The drugs included Nikkomycin Z, an inhibitor of chitin synthase III; Amphotericin B, a polyene that damages cell membranes; tunicamycin, a specific inhibitor of N-glycosylation; sodium orthovanadate (vanadate), resistance to which often indicates defects in glycosylation; Hygromycin B, an aminoglycoside; Echinocandin, an inhibitor of β-1,3-glucan synthesis; and FK

506, a calcineurin inhibitor. Mutations in *FKS1* are known to be hypersensitive to FK506.

Relative to the parental strain (PRY487) three mutant strains (3d-2, 13a-1, and 13d-3) showed increased sensitivity to Nikkomycin Z. Two mutant strains (12a-1 and 12e-1) showed increased sensitivity to Amphotericin B. Only 12e-1 showed increased sensitivity to tunicamycin. None of the mutant strains showed any significant variation from the parent strain when tested for sensitivity to the β-1,3-glucan synthase inhibitor Echinocandin. All five mutant strains (3d-2, 12a-1, 12e-1, 13a-1, and 13d-3) showed increased sensitivity to the aminoglycoside Hygromycin B. Dean (34) demonstrated that abnormal glycosylation results in sensitivity to aminoglycosides, which suggests that all five mutant strains are defective in glycosylation. Four of the synthetic lethal strains showed resistance to vanadate, (3d-2, 12a-1, 13a-1, and 13d-3). Only 13d-3 showed an increased sensitivity to FK 506.

Four mutant strains (3d-2, 12a-1, 13a-1, and 13d-3) exhibit simultaneous growth resistance to vanadate and sensitivity to Hygromycin B, which is characteristic of mutants defective in Golgi-specific glycosylation (35). According to Dean (34) even mutants with defects in the early steps of glycosylation are sensitive to Hygromycin B. Only the 12e-1 mutant strain showed hypersensitivity to tunicamycin. This is also the only mutant that is not resistant to vanadate.

Isolation of Complementing Clone and Database Search. The Nikkomycin Z hypersensitivity of 13a-1 and 13d-3 was used to facilitate the cloning of the complementing insert from the *URA3*-marked *CEN4* library. Initial sequence data obtained from the complementing insert was used to search existing databases. The gene mutated in 13d-3 was identified as *FKS1*, which is identical to *ETG1* (36), *CWH53* (37), *PBR1* (33), and *CND1* (38). The *FKS1* gene encodes a subunit of the *S. cerevisiae* β-1,3-glucan synthase.

For 13a-1, a BLAST query identified a sequence present on chromosome V. Because the library inserts average 9 kb in length, most contain several genes. The entire DNA sequence of this clone was downloaded and analyzed for ORFs. Using subcloned portions of the insert followed by complementation analysis, the mutated gene in 13a-1 was determined to be *ANP1*.

Unlike 13a-1 and 13d-3, 12a-1 showed no sensitivity to Nikkomycin Z. The sensitivity of 12a-1 to Calcofluor white, in addition to the acquisition of the ability to grow on SD without uracil was used to select transformants with a complementing insert. Subcloning and complementation analysis resulted in the identification of the complementing gene in 12a-1 as *SRV2* (*CAP1* and *END14*), which has been implicated in the transmission of cAMP-mediated signals via the *RAS*/adenylyl cyclase pathway (39–41) and more recently shown to bind to the Src homology 3 domain of the actin binding protein, Abp1p. The *SRV2* gene may provide a link between growth signals and the cytoskeleton (42).

Complementation Analysis. Plasmids carrying the isolated complementing gene were used to verify the mutant phenotypes as well as additional members of the complementation group. The plasmid p13d-3, containing the wild-type clone of *FKS1*, was used to transform all five synthetic lethal mutant strains, including 13d-3. The plasmid p13d-3 complemented the sectoring phenotype and drug profile of the original mutant, 13d-3, as well as two additional mutants, 3d-2 and 12e-1. When p12a-1 or p13a-1 were used to transform the putative synthetic lethal strains, only the original mutant strain was complemented for both the sectoring phenotype and drug profile. Thus, we have identified at least three different complementation groups as being essential in combination with a *CHS*3 deletion: *FKS1* (three members), *ANP1* (one member), and *SRV2* (one member).

Cell Wall Composition. The cell wall composition of the mutant strains might suggest the cause of the synthetic lethal interaction with *chs3*. Strains were grown in YPD supplemented with radioactive glucose. Cells were harvested and fractionated (see *Materials and Methods*), and the radioactivity incorporated into the alkali-insoluble fraction and the mannan cell wall fraction was determined (Table 1). The incorporation of label into the alkali-insoluble fraction, containing alkali-insoluble glucan and chitin, was 20–30% lower for the *fks1* mutant strains than for the control strains PRY485 and PRY487. The *fks1* strains showed a level of incorporation of label into mannan comparable to the control strains. Incorporation of label into the alkali-insoluble fraction for the *anp1* strain was approximately equal to the control strains, but incorporation of label into mannan was reduced by 75%. The cell wall composition data for the *srv2* strain was comparable to the control strains. Chitin measurements and their significance are discussed below.

Table 1. Cell wall composition

Strain	Mutation	[14C]glucose incorporated: Alkali-insoluble fraction, %	[14C]glucose incorporated: Mannan, %
PRY487	Wild type	30	18
PRY485	*chs3*	27	21
13d-3	*fks1*	20	21
12e-1	*fks1*	23	17
3d-2	*fks1*	20	21
12a-1	*srv2*	32	19
13a-1	*anp1*	32	5

Results are expressed as percent incorporation of radioacivity from [14C]glucose into cell wall polysaccharides (cpm incorporated per fraction/total counts per min incorporated) × 100.

β-1,3-Glucan Synthase Activity. Glucan is a major component of the yeast cell wall. To better understand the synthetic relationship between our mutants and *chs3*, we measured their β-1,3-glucan synthase activity. Results are summarized in Table 2. Compared with the control strain PRY485, all three members of our *fks1* complementation group, (3d-2, 12e-1, and 13d-3), show a significant reduction in glucan synthase activity, the activity being 15–38% of the control. This finding is consistent with previously published data (33). The *anp1* mutant strain (13a-1) shows a less marked decrease in enzyme activity (66% of the control). Unlike the *fks1* and *anp1* strains, the *srv2* mutant strain (12a-1) shows an increase in enzyme activity, 126% of the control strain.

Other Synthetic Lethal Interactions. Our mutant screen has shown that *chs*3::*LEU*2 is synthetically lethal with *anp1*. The *ANP1* gene is homologous to *VAN1* and *MNN9*. Together, the three constitute a family of genes required for proper Golgi function in *S. cerevisiae* (43). To determine whether or not the other members of this gene family have the same synthetic lethal interaction with *chs*3, heterozygous diploids were constructed with *mnn9* and *van1*. After tetrad dissection, we examined the segregation of markers and the viability of the

Table 2. β-1,3-glucan synthase activity

Strain	Mutation	Activity (% of control)
PRY485	*chs3*	≡100
3d-2	*fks1*	15 (±1)
12e-1	*fks1*	38 (±6)
13d-3	*fks1*	21 (±3)
12a-1	*srv2*	126 (±10)
13a-1	*anp1*	66 (±3)

β-1,3-glucan synthase activity was determined as described, and units of activity were normalized to protein. Activity is expressed as percent of activity of the control strain PRY485. Results are the mean, where $n = 4$, and error is the SEM.

spores. The results show that *chs3* is indeed lethal in combination with both *mnn9* and *van1*.

The synthetic lethality of the *fks1* complementation group along with the Nikkomycin Z hypersensitivity for two of the three mutants suggests that a simultaneous decrease in glucan and chitin is lethal. If true, one would expect *fks1* strains to be lethal in combination with other mutations that result in a loss of chitin synthesized by Chs3p. To test this, strain PRY581 carrying the original mutant *fks1* from 13d-3 (but not the *chs3*::*LEU2* disruption) was mated to the *chs4–4*::*LEU2* strain PRY582, the *chs3*::*LEU2* strain PRY502, and the *csd3/chs6*::*TRP1* strain PRY404. Diploids were dissected and the tetrads were analyzed for segregation of markers and viability. For PRY581/PRY582, 12 double mutant (*chs4*::*LEU2 fksl*) spores were expected. Based on the lack of Leu^+ Nikkomycin Z hypersensitive spores, no double mutants were recovered. Therefore, *fks1* is synthetically lethal in combination with a deletion of *CHS4*. The cross of PRY581 (*fksl*) to PRY502 (*chs3*::*LEU2*) is a reconstruction of the original synthetic lethal strain. For PRY581/PRY502, 12 double mutant spores (*chs3*::*LEU2 fks1*) were expected but none were recovered. This result confirms the synthetic lethality of the double mutant, *chs3 fks1*. The synthetic lethality of *fks1* with both *chs3* and *chs4* supports the idea that a simultaneous reduction in β-(1,3)-glucan and chitin (specifically chitin synthesized by CSIII) is lethal.

For PRY581/PRY404, of the eight double mutant (*csd3/chs6*::*TRP1 fks1*) spores expected, five were viable. Unlike *chs3* and *chs4* strains, *csd3/chs6* deletion strains do have chitin synthase activity *in vitro*. Three tetrads were tested for their ability to grow on YPD plus Calcofluor. Of the 12 spores tested, 10 were Calcofluor sensitive. The excess of Calcofluor-sensitive spores is consistent with the idea that Chs3p is functional in these strains. When analyzed for chitin content the double mutants were found to have the high level of chitin found in *fks1* mutants (Table 3), in striking contrast to *csd3/chs6* mutants that have little, if any, chitin made by the *CHS3* complex.

DISCUSSION

MNN9 Family. The type II membrane proteins Anp1p, Van1p, and Mnn9p, are required for proper Golgi glycoprotein processing in *S. cerevisiae* (44). We found that *chs3* is lethal in combination with deletions of any of the three. Jungmann and Munro (44) have shown that Anp1p, Van1p, and Mnn9p colocalize within the cis Golgi and form two distinct complexes, each complex containing Mnn9p and either Anp1p or Van1p. Both complexes have α-1,6-mannosyltransferase activity. As expected, our *anp1* mutant 13a-1 is resistant to vanadate and sensitive to Hygromycin B. In addition, strain 13a-1 is hypersensitive to Nikkomycin Z, which suggests the need for chitin in these mutants. Determination of β-1,3-glucan synthase activity in the 13a-1 strain showed a reduced level of enzyme activity relative to the control. For this strain, there is a concomitant glycosylation defect along with a decrease in enzyme activity.

Table 3. Chitin levels in *fks1-csd3/chs6* double mutants

Strain	Chitin, nmoles on GlcNAc/mg
PRY224 (WT)	6.7
PRY223 (WT)	8.4
PRY512 (*chs*3)	0.9
PRY514 (*chs*4)	1.1
PRY513 (*csd3/chs6*)	1.9
PRY404 (*csd3/chs6*)	1.9
PRY581 (*fks*1)	23.6
CBY108.1 (*fks*1)	32.4
112.6A (*fks*1, *csd3/chs6*)	47.5
112.10C (*fks*1, *csd3/chs6*)	27.1
112.9B (*fks*1, *csd3/chs6*)	28.1

Chitin levels for cells grown in YPD were measured as described by Bulawa (2) and are expressed as nmoles of acetylglucosamine per mg of cell wet weight. WT, wild type.

Recently, Mondesert *et al.* (45) reported the isolation of morphogenesis checkpoint-dependent (*mcd*) mutants that are defective in growth but have normal actin organization. One of the mutated genes they cloned and identified as *ANP1*. Mondesert *et al.* suggest that N-linked glycosylation is needed not only for the mannoproteins required for cell wall construction during bud emergence but also to direct secretion to the presumptive site of bud emergence and to the emerging bud. Consequently the lethality of *chs3* and *anp1* may be caused by a weakened cell wall, which results from the general decrease in mannan or might be caused by a need for N-glycosylation of protein/proteins as part of a signaling pathway involved in polarization of secretion during the cell cycle.

SRV2. *SRV2* encodes a 526-aa protein that has at least three functional domains. The N-terminal domain (amino acids 1–192) binds to adenylyl cyclase and is necessary and sufficient for the phenotypes associated with activated regulatory gene *RAS. SRV2* is required for *RAS*-activated adenylyl cyclase activity (39) but mutations that make cell viability independent of the production of cAMP do not suppress the lethality of null alleles. Therefore *SRV2* must provide an essential function to the cell that is independent of the production of cAMP. The C-terminal domain is required for normal cellular morphology and response to nutrient extremes (40). The C-terminal domain binds to monomeric actin and has a cytoskeletal regulatory function *in vivo* (46). The middle third of *SRV2* contains a proline-rich region (amino acids 273–286), which has been shown to bind to the Src homology 3 domain of the actin binding protein, Abp1p (42). *SRV2* may play a role in maintaining the integrity of cellular membranes because it is localized to cell membranes (47) and cortical actin patches (42) that are thought to be regions of the cell that are actively growing. Because current speculation favors the concept that cell wall synthesis is controlled by the *PKC1* and the *PBS2-HOG1* regulatory pathways, it will be important to determine where the Srv2p interacts with these two pathways. That it does indeed interact is suggested by the abnormally swollen cell morphology of *srv2* null mutants (40). The cell wall composition analysis shows that cell walls of the 12a-1 strain carrying *srv2* are similar to those of the control strain, suggesting that the synthetic lethality is not caused by a simple decrease in wall polymers.

We have shown previously (4) that under certain growth conditions, specifically growth on YPD, the double mutant *chs2 chs3* is lethal. If the Srv2p is required for proper localization and/or functioning of the Chs2p, strains carrying a mutated *srv2* gene would require a functional *CHS3* gene for viability. Another possible explanation for the data would be that Srv2p is required for proper localization and functioning of the 10-nm filaments encoded by the cell division cycle genes *CDC3, CDC10, CDC11*, and *CDC12*. The results of our unpublished tetrad analyses, done for crosses of a *chs3* deletion strain with *cdc3, cdc10, cdc11*, or *cdc12,* suggest that chs3 is lethal in combination with *cdc3, cdc11*, and *cdc12* but not with *cdc10*. This result is interesting and suggests that the principle function of Cdc10p may be binding of the Chs3p complex to the septin ring, and that it otherwise may be dispensable.

FKS1. We were not surprised to find three *fks1* mutants among our synthetically lethal isolates, because lack of both chitin and part of the cell wall β-1,3-glucan should be fatal. *FKS1* encodes a subunit of one of the two enzymes that synthesize 1,3-β-glucan, a major structural component of the cell wall. Mutations in this gene have been isolated in other screens designed to identify cell wall alterations, e.g., hypersensitivity to FK506 (48), resistance to Echinocandins (49, 50),

and sensitivity to Calcofluor white (37). As expected, all three of our *fks1* mutants have reduced β-1,3-glucan synthase activity relative to the control strain PRY485. The residual enzyme activity is thought to be catalyzed by *FKS*2, a homolog of *FKS1* (Fks2p is 88% identical to Fks1p). In continuing this investigation we found, as expected, that deletion of *CHS4*, a probable second subunit of the Chs3p enzyme complex, is also lethal in *fks1* mutants.

At least seven known *Saccharomyces* mutations that weaken the cell wall lead to substantial increases in the level of chitin deposited in the lateral wall, probably by Chs3p (51). *Fks1* and *gas1* (52) mutants are among the most prominent strains showing increased chitin synthesis, with chitin levels as much as an order of magnitude higher than those found in wild-type cells. Deposition of high tensile strength chitin fibers in the wall may be a physiologically important "stress response" programmed into the cell, a stereotyped response that probably should be differentiated from "delocalized" chitin synthesis. The latter frequently is associated with disorganization of the actin cytoskeleton (53).

Although nothing is known about the mechanism of stress response chitin synthesis, one would assume that it proceeds by the normal pathways of Chs3p targeting. This pathway involves exit of Chs3p from the endoplasmic reticulum under the control of Chs7p (17), movement to chitosomes, and the plasma membrane mediated by Csd3/Chs6p (16) and probably Chs5p, and then recycling from the plasma membrane to targeted areas such as the site of incipient bud formation and the lateral wall through an endocytic route in vesicles that contain the syntaxin Tlg1p (54).

In spite of strong evidence supporting the operation of this pathway under normal conditions, the elevated chitin level in the *csd3/chs6 fks1* double mutant demonstrates that the normal role of *CSD3/CHS6* is being bypassed where increased chitin synthesis is required. Clearly an alternate means of transport and activation is being used. Definition of this pathway and its regulation represents a new and exciting area of investigation.

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